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Abstract

Two simple methods are presented that allow positive identification of the accuracy and precision of the microsphere technique and a quick verification of sphere entrapment in tumor vessels. A known flow of Ringer's solution from a motor-driven syringe is perfused through the rat's isolated systemic circulation from left ventricle to right atrium and collected in a funnel. Using this preparation, total blood flow in rats measured with radioactive microspheres injected into the left ventricle was 97% of actual flow. The coefficient of variation (standard deviation/mean) of the microsphere measurements was 0.22. In the same preparation, non-entrapment of microspheres in subcutaneous tumor nodules grown on a hind limb could be measured from the difference in counts collected in venous effluent before and after placement of a tourniquet proximal to the tumor. For example, in two types of transplantable carcinoma, we found non-entrapment of less than 0.1% of the injected microspheres. Such a shunt would correspond to less than 10% of microspheres entering a typical tumor nodule and, in turn, less than 10% underestimation of true flow to the tumor. These two techniques may be helpful to other investigators in testing the accuracy of microsphere methods in various small animal tumor models.

Key words: Tumor blood flow; Microsphere technique

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INTRODUCTION

Tumor blood flow is of great importance in determining the response of neoplastic tissue to several treatment modalities. It is known that oxygenated cells are more sensitive to radiation than hypoxic cells (Casarett, 1964). In turn, larger tumors, which tend to have reduced blood flow, are more radioresistant (Mantyla, 1979). On the other hand larger and/or poorly perfused tumors that cannot dissipate heat effectively may be especially vulnerable to local hyperthermia therapy (Alfieri et al., 1975; Babbs and DeWitt, 1981; Johnson, 1978; LeVeen et al., 1976; Storm, 1979). The selectivity of intra-arterial chemotherapy may be dependent upon the relative vascularity and perfusion of the tumor and, obversely, drugs that alter the ratio of tumor to normal tissue perfusion may enhance the therapeutic indices of certain chemotherapeutic agents (Voorhees, 1982; Mattsson et al., 1980). The importance of tumor perfusion in natural history and treatment of cancer is emphasized in several monographs and reviews (e.g. Peterson, 1978).

Some of the methods for measuring tumor blood flow that have been previously reported include direct measurement of venous outflow in organs totally replaced by tumor (Gullino and Granthan, 1961), the isotope wash-out method (Moiler and Boijsen, 1975), the dye dilution method (Goldrace and Sylven, 1959), a thermal circulation index (Kruuv et al., 1967), and, more recently, the microsphere technique (Reneman and Verheyen, 1977). Radioactive microspheres have been used with increasing frequency for determining tumor blood flow in small animals (Bartium et al., 1974; Gjedde et al., 1977; Hafstrom et al., 1980; Song, 1981). This technique is especially attractive in that it is simple to use and permits measurement of flow to multiple organs and tissues at the same time (Bartium et al., 1974; Gjedde et al., 1977). Results of this technique compare favorably with those of the dye dilution and clearance methods in dogs, monkeys, and rabbits (Bartium et al., 1974; Delaney and Grim, 1964; Hoffbrand and Forsyth, 1969). Some care is required, however, to adapt this technique for use in small animals such as rats. The presence of a relatively large catheter, for example, can cause alteration in the cardiac output (Gjedde et al., 1977). Measured organ blood flows, and especially tumor blood flows, may be more dependent upon the diameter of microspheres injected in small animals than in large animals (Heyman et al., 1977; Jirtle et al., 1978; Reneman et al., 1977).

In particular, the microcirculation of solid tumors in which the microspheres are trapped differs from that of normal tissues in the abundance of sinusoidal capillary beds. These tumor capillary sinusoids are broader, longer, and are separated by larger intercapillary distances than normal capillaries. (Folkman, 1976; Ide et al., 1939; lntaglietta et al., 1977; Vaupel, 1977). This general pattern of tumor vascularity has been observed in a variety of systems, including rat myelosarcoma (Habighorst, 1977), transplanted Brown-Pearce rabbit epithelioma (Ide et al., 1939), hamster malignant neurilemoma (Eddy and Casarett, 1976), and rat BA 1112 sarcoma implants (Gross, 1979). There is the distinct possibility, therefore, that tumor vessels may not trap microspheres as efficiently or in the same proportions as do normal tissues. For these reasons, we devised two simple experiments: one to check the accuracy of the microsphere technique against an accurate and fixed standard, namely a motor-driven syringe, and the other to check for non-entrapment of microspheres in a particular experimental tumor type.

METHODS

Tumors

Inbred male Harlan Fischer rats were housed individually in a temperature-controlled room with a 12 hr light and dark cycle. Tumor transplant was performed on rats aged six weeks and experiments were performed at eight to nine weeks of age. The two tumor types used were carcinomas that had the same origin but differed greatly in their vasculatures. Both of them diverged from a jaw tumor which first appeared in a rat being fed the carcinogen FAA, (N-2-fluronenylacetamide) (Kioppel and Morre, 1980). In order to differentiate the two we named then the "hard" and "soft" tumors.

To transplant these tumors onto the left thighs of the rats, a tumor was excised from a donor rat and minced into very small pieces (~1 mm each). Two to four pieces were than implanted subcutaneously into the left thigh using a trocar needle. A growing period of two to three weeks was permitted.

Animal Preparation

Each rat was first anesthetized with ketamine (0.2 g/kg i.m.) and then was heparinized (0.1 mg/rat). Two PE 50 polyethelene catheters filled with heparinized Ringer's solution were inserted, one into the right femoral artery and the other into the left ventricle via the right carotid artery. To create a test situation in which true systemic perfusion would be known with certainty, we established an isolated systemic circulation (Figure 1).

Through a midline thoracotomy, a 16-gauge plastic cannula, connected via tubing to a Harvard infusion pump, was placed directly into the left ventricle through a stab wound in the cardiac apex. The tip of the cannula had been flared by heating so that it could be easily secured in the apex of the left ventricle by a purse-string suture. Heparinized, oxygenated Ringer's solution was perfused intermittently into the left ventricle and through the systemic circulation using a motor-driven 60-ml syringe at a rate of 32 ml/min. This rate was verified before and after the experiment by timed collection in a graduated cylinder. The right atrium was incised to allow the perfusate to leave the venous system; the perfusate was collected in a funnel. The pulmonary arteries were ligated to prevent recirculation.

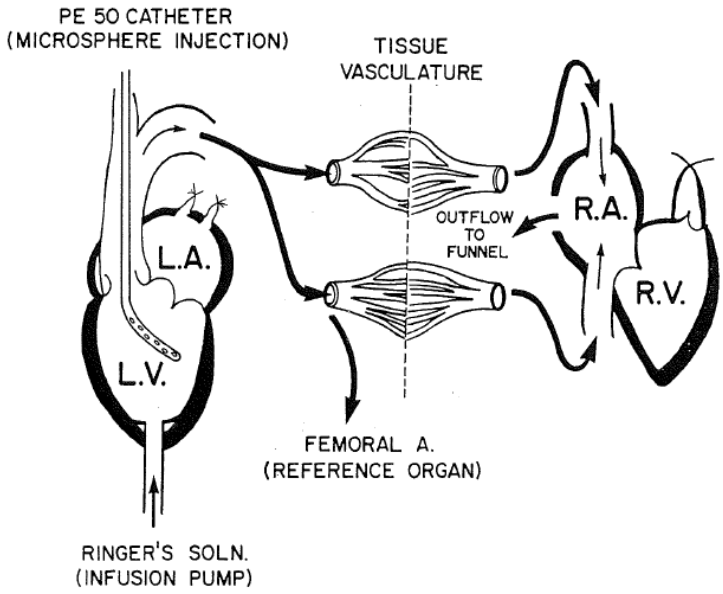

FIGURE 1. Schematic representation of the set-up. (L.A., R.A., LV., and R.V. represent the left and right chambers of the heart.)

Microsphere Preparation

Microspheres used for the studies were manufactured by the 3M Company (Minneapolis, MN). These spheres had an average diameter of 15 μm and were suspended in 10% dextran and 0.5% Tween 80 with a concentration of 5 mCi per 50-ml solution. The four labels used were Ce-141, Sr-85, Nb-95, and Sc-46. Each was sonicated for 30 min prior injection to ensure disaggregation and uniform suspension of the spheres (Heyman et al., 1977). The volume injected each time was 0.3 ml containing approximately 100,000 spheres.

Accuracy Study

To determine the accuracy of the microsphere technique, the known flow was established with the Harvard infusion pump. The flow rate was then measured in each preparation on four successive trials, using microspheres with the Ce, Sr, Nb, and Sc labels; it was compared to the known flow rate through the system of 32 ml/min. A reference sample of perfusate was collected through the femoral catheter 5 sec before the first injection and continued for 1.5 min. The reference sample was allowed to drip into a vial and the volume (approximately 1 ml) was measured after counting to determine the reference flow rate. Based on the assumption that the microspheres are mixed uniformly with the perfusate at the time of injection and are distributed to the tissues in the same proportions as the vascular flow, the flow rate to any tissue sample can then be determined by comparing the activities in the tissue sample (cpm) to that of the reference sample, according to the proportion:

tissue flow rate/reference flow rate = tissue activity/reference activity.

For the accuracy study, we calculated the total flow rate through the rat using the microsphere method, and so the tissue activity was taken as the total activity injected. Microspheres remaining in the left ventricular catheter were drawn back into a vial and the total activity injected into the system was determined by subtracting the remaining activities from the activities of the prepared standards. All samples collected were counted in a 1.5-in. diameter NaI well counter connected to a multichannel analyzer (Canberra).

To assess the accuracy and precision of the microsphere technique in this test system, we plotted histograms of 36 measured flow values obtained from nine normal rat preparations and compared this distribution to the true flow value (32 ml/min). As defined in ***Biometry*** (Sohal and Rahlf, 1969), accuracy is taken as the closeness of a measured value to the true value, and precision is taken as the closeness of the repeated measurements to one another. Hence we interpreted "accuracy" as being related to the difference between the mean measured value and the true value, and "precision" as being inversely related to the standard deviation of the measured values.

Non-entrapment Study

Both normal and tumor-bearing rats used in this study were prepared similarly to those in the accuracy study with the exception that no catheterization of the femoral artery was performed. Instead, total effluent was collected directly from the right atrium. To minimize leakage of microspheres from the opened chest wall, surgical clamps were applied to the wound edges. The prepared rat was placed in the prone position over a wire mesh, and perfusate was collected from the right atrium through the open thoracotomy with a funnel. Volume fractions of effluent were collected over the entire experimental period, which extended through four to six microsphere injections and rinses.

If a significant number of 15 μm spheres passed through the tumor vessels without being entrapped, they would appear in the fluid collected from the right atrium. To determine if such activity was indeed coming from the tumor-bearing leg, we applied a tourniquet to the leg, between the tumor and the heart on alternate trials. The whole process was then repeated six times on each rat, with the tourniquet applied between the tumor and the heart on every other trial. With this system, non-entrapment of microspheres in the tumor would be revealed by decreased radioactivity in the collected venous perfusate following microsphere injections with the tourniquet in place. The percent of injected microspheres collected in the venous effluent was determined by adding the entire radioactivity collected with the leg tied or untied and dividing by the total radioactivity injected.

To evaluate the sensitivity of this technique to detect non-entrapment of microspheres, a series of normal rats was also studied. In these animals, a leg tourniquet was alternately applied and released and the difference in venous counts collected was noted to provide an estimate of the experimental variation. In these animals, which did not have implanted tumor nodules, one would expect a negligible difference in the number of spheres collected in the venous effluent.

To clarify the data analysis, two differently labeled spheres were used, one for tied and other for untied conditions.

RESULTS

Of the 36 measurements made in the accuracy study using the microsphere technique, an average of 32.6 ± 7.3 ml/min (S.D.) was obtained. Infusion pump output measured with a graduated cylinder was 31.7 ± 0.4 ml/min (S.D.). Figure 2 gives the frequency distribution of the ratios of microsphere flow to actual flow (pump value). In this histogram, actual flow (31.7 ml/min) is represented by 1.0 on the horizontal axis. Precision of the microsphere measurements is indicated by the dispersion of the distribution about its mean value.

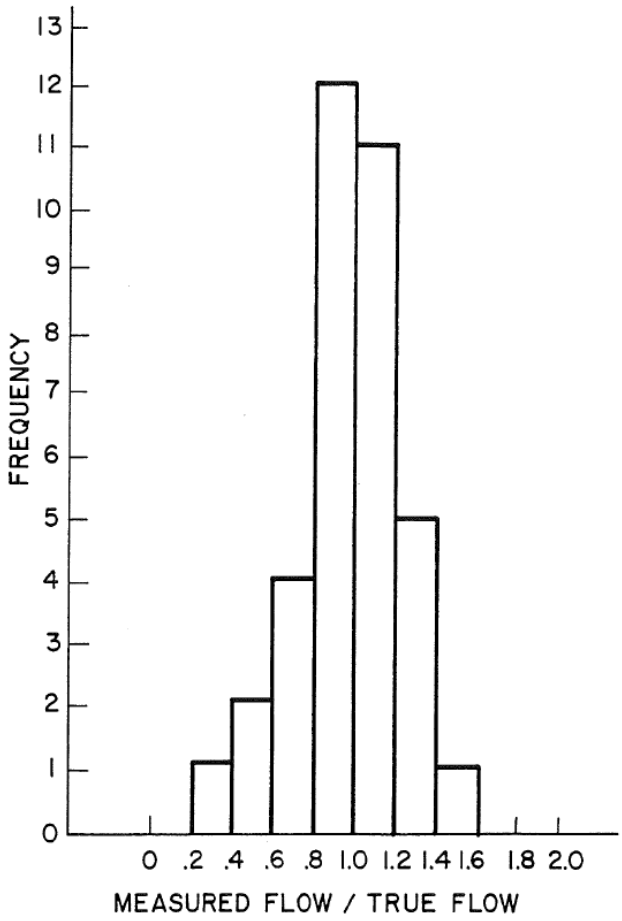

FIGURE 2. Histogram showing the frequency distribution of the microsphere measured flow rate versus that measured directly from the pump. The mean value of the distribution is 97% of the true value. The coefficient of variation of the data (standard deviation/mean) is 0.22.

Percent leakage of microspheres through systemic capillaries from all the rats studied is presented in Table 1. In Table 1, each line represents the average percent leakage with limbs either tied or untied in a single rat. Non-entrapment of microspheres in the normal rat's leg, which is taken as the difference in leakage for the two cases (untied-tied), averaged 0.02%. In this experiment, a positive difference would reveal non-entrapment of microspheres in the tumor vascular bed. However, the observed mean difference was clearly not significantly different from zero. Similar results were obtained for the two tumor types studied, suggesting minimal shunting of microspheres through the vasculature of these tumors.

TABLE 1 Percent Non-entrapment of Microspheres Under Various Test Conditions

	Percent leakage with leg tourniquet		Percent difference
Group	Untied	Tied	Untied—Tied
Normal rats	1.34	1.43	−0.09
	1.65	1.70	−0.05
	1.38	1.28	+0.10
	1.23	0.94	+0.29
	1.27	1.22	+0.05
	0.85	1.04	−0.19
	1.22	1.21	+0.01
Mean ± S.E.	1.28 ± 0.09	1.26 ± 0.09	+0.02 ± 0.06
Soft tumor	1.63	1.85	−0.22
	1.26	1.51	−0.25
	0.98	1.06	−0.08
	1.16	1.10	+0.06
Mean ± S.E.	1.26 ± 0.14	1.38 ± 0.19	−0.12 ± 0.07
Hard tumor	1.27	1.17	+0.10
	0.91	0.94	−0.03
	1.51	1.66	−0.15
	1.06	1.00	+0.06
Mean ± S.E.	1.19 ± 0.13	1.19 ± 0.16	−0.01 ± 0.06

DISCUSSION

Ever since Rudolph and Heymann (1967) introduced the use of microspheres to measure cardiac output in 1967, the method has been adapted to measure regional blood flow rates in various organs and tissues (Reneman and Verheyen, 1977). Validation of the technique is usually performed by comparison with other existing techniques. For example, in a study conducted by Archie and coworkers (1973) non-simultaneous injection of indocyanine green dye and microspheres to measure cardiac output in lambs resulted in a 20% or greater difference of the two in 62% of the data. Rudy and coworkers (1973) obtained similar results: 65% of 30 points are within 20% agreement in monkeys. Bartium's group (1974) found a 0.843 regression coefficient microsphere and green dye cardiac output in rabbits.

When the microsphere technique was compared to the clearance method, using radioactive potassium (K-42) in dogs, a difference of 5.4% was observed (Delaney and Grim, 1964). Rudy (1973) performed a cardiopulmonary bypass on a group of monkeys. The extracorporeal perfusion circuit was a roller pump set at a rate of 200-250 ml/kg per minute to pump oxygenated rhesus blood through the live monkeys. By using the cardiopulmonary bypass method, which has been considered a more direct and accurate means to establish a reference value of flow, Rudy and coworkers found the microsphere technique to be accurate to ± 15%, as compared to the roller pump.

In the accuracy study described in this paper we were able to demonstrate a 3% difference between the mean calculated flow and actual flow rate as provided by the infusion pump, thus suggesting that the microsphere technique can be dependable, on the average, in small animals and is capable of measuring true physiological differences between groups of animals. However, a considerable variation in the data obtained from these small animals is indicated by a 0.22 coefficient of variation (standard deviation/mean). This variability is comparable to that observed by Hossmann et al. (1978), who studied cerebral cortical blood flow in cats using radioactive microspheres and found that the coefficient of variation in the control group was approximately 0.36. Such results indicate that repeated measurements are necessary in order to obtain a good estimate of the true mean flow value under any given experimental condition. In short, the method is accurate but not especially precise.

The basic assumption for the non-entrapment study was that if the spheres used were so small that they leaked from the tumor's capillary beds into the venous system, fewer spheres should have appeared in venous blood when the tourniquet was in place. Non-entrapment of microspheres in tumor capillaries, but not in normal capillaries, is a reasonable possibility in view of the previous reports of abnormally large capillary sinusoids in several experimental and naturally occurring tumor types (Folkman, 1976; Ide et al., 1939; lntaglietta et al., 1977 Vaupel, 1977). Moreover, a significant amount of non-entrapment has been reported when small-sized spheres were used to measure normal tissue flow rate (Archie et al., 1973; Marcus et al., 1976; Ring et al., 1962). Thus, by comparing the venous counts with the circulation to the tumor-bearing leg first occluded and then unoccluded, one can determine whether the spheres used were too small for entrapment in a particular type of tumor. This method can be used for

selecting the best sphere size to use and as a quick way to verify whether the chosen sphere size is adequate for a particular measurement.

In the control observations in animals without tumor (Table 1), repeated measurements of venous effluent activity agreed to within 0.01 to 0.3% of total injected activity in any given animal, and on the average was only 0.02 ± 0.06% for the population. Thus, by implication, the experiment is capable of detecting non-entrapment of less than 0.1% of injected microspheres in tumor nodules grown in a small test group of animals. For extremely small tumors or tumors with very low flow rate, this method would not be very sensitive. However, for a typical 10 g tumor with flow rate about 0.1 ml/min/g, 10% non-entrapment can be easily recognized by comparison with the control, in a rat with a cardiac output of 100 ml/min. With the use of a larger sample size and longer counting times, the sensitivity of the non-entrapment test can be further improved.

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